APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2010, p. 275–282 Vol. 76, No. 1
0099-2240/10/$12.00 doi:10.1128/AEM.02154-09

"*Candidatus* Curculioniphilus buchneri," a Novel Clade of Bacterial Endocellular Symbionts from Weevils of the Genus *Curculio*▽†

Hirokazu Toju,[1] Takahiro Hosokawa,[1] Ryuichi Koga,[1] Naruo Nikoh,[2] Xian Ying Meng,[1] Nobutada Kimura,[1] and Takema Fukatsu[1]*

National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan,[1] and Department of Liberal Arts, The Open University of Japan, Chiba 261-8586, Japan[2]

Received 7 September 2009/Accepted 26 October 2009

Here we investigated the bacterial endosymbionts of weevils of the genus *Curculio*. From all four species of *Curculio* weevils examined, a novel group of bacterial gene sequences were consistently identified. Molecular phylogenetic analyses demonstrated that the sequences formed a distinct clade in the *Gammaproteobacteria*, which was not related to previously known groups of weevil endosymbionts such as *Nardonella* spp. and *Sodalis*-allied symbionts. In situ hybridization revealed that the bacterium was intracellularly harbored in a bacteriome associated with larval midgut. In adult females, the bacterium was localized in the germalia at the tip of each overiole, suggesting vertical transmission via ovarial passage. Diagnostic PCR surveys detected high prevalence of the bacterial infection in natural host populations. Electron microscopy identified the reduced cell wall of the bacterial cells, and the bacterial genes exhibited AT-biased nucleotide composition and accelerated molecular evolution, which are suggestive of a long-lasting endosymbiotic association. On the basis of these results, we conclude that the novel endocellular bacteria represent the primary symbiont of *Curculio* weevils and proposed the designation "*Candidatus* Curculioniphilus buchneri." In addition to "*Ca.* Curculioniphilus," we identified *Sodalis*-allied gammaproteobacterial endosymbionts from the chestnut weevil, *Curculio sikkimensis*, which exhibited partial infection frequencies in host insect populations and neither AT-biased nucleotide composition nor accelerated molecular evolution. We suggest that such *Sodalis*-allied secondary symbionts in weevils might provide a potential source for symbiont replacements, as has occurred in an ancestor of *Sitophilus* grain weevils.

Diverse insects are obligatorily or facultatively associated with endosymbiotic bacteria, many of which provide a set of "tool kits" consisting of various genes that confer benefits to their host insects (5, 20, 30), such as supply of essential nutrients (1, 39), tolerance to high temperature (29), resistance to parasites and pathogens (33, 37), alteration of food plant range (16, 45), synthesis of toxic substances (21, 48), enhancement of energy metabolism (13), etc. On the other hand, some endosymbiotic bacteria are rather parasitic and affect their host insects negatively, either by reducing the host survival and fecundity or by manipulating the host's reproduction in a selfish manner (3, 46). The knowledge of such ubiquitous microbial associates is pivotal for understanding the ecology, adaptation, and evolution of insects.

The weevil superfamily Curculionoidea (Insecta: Coleoptera) represents the most species-rich animal group, embracing over 60,000 described species (27). Early histological studies reported that many, if not all, of them possess specialized symbiotic organs and harbor bacterial endosymbionts therein (4, 5, 26, 31, 32, 34, 38). Despite the potential diversity of weevil endosymbionts, modern microbiological characterization has been performed on a quite limited number of weevil groups: mostly weevils of the family Dryophthoridae and a few species from the subfamilies Molytinae and Cryptorhynchinae. A clade of gammaproteobacterial endosymbionts, *Nardonella* spp., has been identified from diverse dryophthorid weevils of the genera *Cosmopolites*, *Matamasius*, *Rhynchophorus*, *Scyphophorus*, *Sphenophorus*, and *Yuccaborus* (24); two molytine weevils of the genus *Hylobius* (6); and a cryptorhynchine weevil, *Euscepes postfasciatus* (15). The evolutionary origin of *Nardonella* is thought to be quite ancient: dating back to an ancestor of those weevil groups some 125 million years ago (6). Meanwhile, within several dryophthorid lineages, *Nardonella* has been lost and replaced by different endosymbiotic bacteria: the grain weevils of the genus *Sitophilus* harbor *Sodalis*-allied gammaproteobacterial endosymbionts (14), and the weevils of the genera *Trigonotarsus* and *Diocarandra* are associated with a distinct and unnamed gammaproteobacterial clade (24).

The acorn or seed weevils of the genus *Curculio* (Curculionidae: Curculioninae) are characterized by their extremely elongated snouts (see Fig. 2A), which female insects utilize for boring a hole on their host plant seed and deposit an egg into the hole. Larvae feed on the content of the seed, escape the seed upon maturity, and pupate in the soil (17, 25, 28). Some *Curculio* species are notorious agricultural pests, represented by the chestnut weevil, *C. sikkimensis*, which damages chestnut production (19). In two species of *Curculio* (= *Balaninus* Germar 1817) weevils, Buchner (4) observed that filamentous bacteria are harbored in an aggregation of tiny bacteriocytes located at the posterior convolution of the midgut in young larvae. Since the early histological description, however, the

* Corresponding author. Mailing address: National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan. Phone: 81-29-861-6087. Fax: 81-29-861-6080. E-mail: t-fukatsu@aist.go.jp.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 30 October 2009.

microbiological nature of the *Curculio* endosymbiont has been elusive to date.

In this study, we examined Japanese *Curculio* weevils for their endosymbiotic bacteria and identified previously unknown types of weevil endosymbionts: gammaproteobacterial primary symbionts constituting a novel endosymbiont clade and *Sodalis*-allied gammaproteobacterial secondary symbionts.

MATERIALS AND METHODS

Insect materials. Table 1 summarizes the insect materials used in this study. Fagaceous seeds collected in summer of 2006 to 2008 were kept in the laboratory, from which final instar larvae of *C. sikkimensis*, *C. dentipes*, and *C. robustus* that emerged for overwintering and pupation were harvested. About a half of the larvae of *C. sikkimensis* were individually introduced into plastic cases filled with soil, from which emerging adult insects were collected in next spring. Adult insects of *C. dentipes* and *C. robustus* were obtained in the same way. These adult insects were preserved in acetone for DNA analyses (9). The other half of the larvae of *C. sikkimensis* were immediately preserved in acetone upon collection. Some of the larvae and adults of *C. sikkimensis* were kept alive and subjected to in situ hybridization and electron microscopy. We also collected camellia seeds to obtain the larvae of *C. camelliae* (44), which were reared in the soil until eclosion.

DNA extraction, cloning, and sequencing. From each of the acetone-preserved larvae, gut was dissected in 70% ethanol. From each of the acetone-preserved adults, gut plus ovary/testis was dissected. These dissected tissue samples were individually subjected to DNA extraction using a QIAamp DNA minikit (Qiagen). From the DNA samples, the following bacterial genes were amplified by PCR: a 1.5-kb fragment of the bacterial 16S rRNA gene with primers 16SA1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16SB1 (5′-TACGGYTACCTTG TTACGACTT-3′) (11), a 0.9-kb fragment of the bacterial *groEL* gene with primers groEL2F (5′-ATGGGBGCTCAAATGGTKAAA-3′) and groEL2R (5′-CTCTTTCATTTCAACTTCNGTBGCA-3′), and a 1.3-kb fragment of the *Sodalis*-allied bacterial *groEL* gene with GroELSod200F (5′-GAACATGGGCG CCCAGATGGTG-3′) and GroELSod1480R (5′-CGGGTTACCTTGGTCGG ATCCAG-3′). The PCR products were subjected to cloning, up to eight clones for each of the samples were genotyped by restriction fragment length polymorphism (RFLP), and multiple clones representing each of the identified genotypes were sequenced using BigDye Terminator cycle sequencing kit (Applied Biosystems) and an ABI 3130 DNA sequencer (Applied Biosystems).

Molecular phylogenetic analysis. Multiple alignments of the DNA sequences were generated using the program ClustalW (43). For the alignments, the best-fit substitution models were selected using the program Kakusan3 (42). Bayesian phylogenies were inferred using the program MrBayes v3.1.2 (36). Maximum likelihood phylogenies were estimated using the program PhyML v3 (12) with SPR branch-swapping and 10 neighbor-joining starting trees. The reliability of each clade was evaluated by 100 bootstrap replicates.

Diagnostic PCR. From three localities in Japan, Mt. Tsukuba, Hacchodaira, and Shohbara, 15 adult females and 15 adult males of *C. sikkimensis* were collected and individually subjected to DNA extraction and diagnostic PCR (Table 1). The quality of the DNA samples was evaluated by amplification of a 0.1-kb fragment of elongation factor 1α (EF1α) gene of the host insect with primers RferEF1a546F (5′-TCCAGCCAACATCACCACTG-3′) and RferEF1a617R (5′-CCGGGTAC AGCTTCTTGCA-3′). The presence of *Nardonella* spp. was checked by amplification of a 0.3-kb fragment of the 16S rRNA gene with primers 16SA1 and Nar16S250R (5′-CTCATCWWWKGRYRTAAGGTT-3′), wherein a DNA sample of the dryophthorid weevil *Rhynchophorus ferrugineus* was used as a positive control. The infection frequencies of the novel symbiont were examined by amplification of a 0.5-kb fragment of a 16S rRNA gene with primers 16SA1 and PrmSikk464R (5′-TGCATTGTTGCCTTCTTTCCTA-3′) and a 0.5-kb fragment of the *groEL* gene with primers GroPrmSik1000F (5′-TACTACTACTAT TATTGATGGAGC-3′) and GroEL2R (5′-CTCTTTCATTTCAACTTCNGTB GCA-3′). The infection frequencies of the *Sodalis*-allied symbiont were examined by amplification of a 0.5-kb fragment of the 16S rRNA gene with primers Sodalis370F (5′-CGRTRGCGTTAAYAGCGC-3′) and 16SSod590R (5′-AACAGACCGCCTGCGTACG-3′) and a 0.5-kb fragment of the *groEL* gene with primers GroELSod200F (5′-GAACATGGGCGCCCAGATGGTG-3′) and GroELSod500R (5′-CCSGAACCCTCTTCCACGGTGATG-3′). For each of the symbionts, two genes were targeted to confirm the reproducibility of the symbiont detection. A DNA sample of *C. sikkimensis*, from which 16S rRNA

TABLE 1. *Curculio* weevils used in this study, their symbiont infections, and nucleotide sequence accession numbers for the symbiont genes

Insect species or sample code	Locality[a]	Latitude and longitude	Host plant	Sex/stage of insect[b]	% (no. confirmed/total) identified by diagnostic PCR			Accession no. (gene)	
					Curculioniphilus[c]	*Sodalis*	*Nardonella*	*Curculioniphilus*[c]	*Sodalis*
C. sikkimensis									
TKB	Mt. Tsukuba, Ibaraki	36°13′N 133°12′E	*Quercus serrata*	Female	100 (15/15)	20 (3/15)	0 (0/15)	AB514502 (16S rRNA)	AB514505 (16S rRNA)
				Male	100 (15/15)	27 (4/15)	0 (0/15)	AB514497 (*groEL*)	AB514501 (*groEL*)
HCD	Hacchodaira, Kyoto	35°15′N 135°50′E	*Quercus crispula*	Female	87 (13/15)	0 (0/15)	0 (0/15)	AB514503 (16S rRNA)	
				Male	87 (13/15)	0 (0/15)	0 (0/15)		
SHB	Shohbara, Hiroshima	34°52′N 133°12′E	*Quercus serrata*	Female	100 (15/15)	47 (7/15)	0 (0/15)	AB514504 (16S rRNA)	AB517595 (16S rRNA)
				Male	100 (15/15)	47 (7/15)	0 (0/15)		
Total					96 (86/90)	23 (21/90)	0 (0/90)		
C. camelliae	Kyohgatake, Nagasaki	32°59′N 130°05′E	*Camellia japonica*	Female				AB507716 (16S rRNA) AB514498 (*groEL*)	
C. dentipes	Shimoda, Shizuoka	34°40′N 138°59′E	*Quercus phillyraeoides*	Female				AB507714 (16S rRNA) AB514499 (*groEL*)	
C. robustus	Nokonoshima, Fukuoka	33°37′N 130°19′E	*Quercus acutissima*	Female				AB507715 (16S rRNA) AB514500 (*groEL*)	

[a] All localities are in Japan.
[b] Either the adult female or adult male.
[c] Name proposed in this study.

genes of both the novel symbiont and the *Sodalis*-allied symbiont had been cloned and sequenced, was used as a positive control.

In situ hybridization. Gut of larvae and ovary of adult females of *C. sikkimensis* were dissected in 70% ethanol and immediately preserved in Carnoy's solution (ethanol-chloroform-acetic acid at 6:3:1). After an overnight fixation, these tissues were treated with 6% hydrogen peroxide in 80% ethanol for a week to eliminate autofluorescence (23). Then the samples were thoroughly washed with 100% ethanol and subjected to whole-mount in situ hybridization. An oligonucleotide probe specific to the 16S rRNA sequence of the novel symbiont of *C. sikkimensis*, PrmSikk464R (5′-TGCATTGTTGCCTTCTTTCCTA-3′), with an Alexa 555 fluorescence dye labeled on the 5′ end, was used. The tissues were incubated in a hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, and 30% formamide) containing 100 nM probe and 500 nM SYTOX green (Invitrogen). After an overnight incubation at room temperature, the tissues were thoroughly washed in Dulbecco's phosphate-buffered saline (Sigma-Aldrich), mounted in Slowfade Gold antifade reagent (Invitrogen), and observed under an epifluorescent microscope (Axiophot; Carl Zeiss) and a laser-scanning confocal microscope (LSCM Pascal5; Carl Zeiss).

Electron microscopy. The bacteriome-associated midgut sections were dissected and prefixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C overnight and postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 90 min. After dehydration through an ethanol series, the tissues were embedded in Spurr resin (Nisshin-EM). Ultrathin sections were made on an ultramicrotome (Ultracat-N; Leichert-Nissei), mounted on collodion-coated copper meshes, stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (H-7000; Hitachi).

RESULTS

Bacterial 16S rRNA gene sequences from *Curculio* weevils. A 1.5-kb fragment of the bacterial 16S rRNA gene was amplified by PCR and cloned from DNA samples of *C. sikkimensis* of different geographic origins. RFLP genotyping of the 16S rRNA gene clones from each of the insects was performed, and two major genotypes, A-type and B-type, were identified from four insects of the Tsukuba population and seven insects of the Shohbara population, while only the A-type was detected in an insect of the Hacchodaira population. An insect sample from each of the species *C. camelliae*, *C. dentipes*, and *C. robustus* was also subjected to the analysis, and only the A-type was detected as a major clone. In addition to the A-type and B-type clones, several minor genotypes were also identified from those samples.

We sequenced the A-type clones from insects of three *C. sikkimensis* populations. The 1,463-bp sequences were almost identical to each other (99.7% [1,459/1,463] to 99.8% [1,460/1,463] identities). We also sequenced the A-type clones from *C. camelliae*, *C. dentipes*, and *C. robustus*. The 1,466- to 1,471-bp sequences were similar to each other (94.9% [1,399/1,474] to 97.8% [1,441/1,473] identities) and also similar to the sequences from *C. sikkimensis* (95.4% [1,400/1,467] to 98.0% [1,441/1,471] identities). BLAST searches with the A-type sequence from *C. sikkimensis* as the query retrieved gammaproteobacterial sequences. The top hit sequence was *Sodalis glossinidius*, an endosymbiont of the tsetse fly *Glossina morsitans*, with moderate sequence similarity (93.4% [1,370/1,467] identity; accession no. AP008232).

We also sequenced the B-type clones from two *C. sikkimensis* populations. The 1,454-bp sequences were almost identical to each other (99.4% [1446/1454] identity). BLAST searches with the B-type sequence from *C. sikkimensis* as the query retrieved gammaproteobacterial sequences. The top hit sequence was an endosymbiont of the dryophthorid grain weevil *Sitophilus oryzae* with high sequence similarity (99.0% [1,439/1,455] identity; accession no. AF548137).

Phylogenetic placement of the bacterial 16S rRNA gene sequences from *Curculio* weevils. The A-type and B-type 16S rRNA gene sequences were subjected to molecular phylogenetic analysis with 16S rRNA gene sequences of gammaproteobacterial representatives (Fig. 1). The A-type sequences obtained from *Curculio* spp. constituted a monophyletic group with high statistical supports. Within the group, the sequences from different populations of *C. sikkimensis* formed a robust clade. The bacterial clade was distinct from any of the weevil endosymbiont groups previously described: *Nardonella* spp. universally detected from dryophthorid, molytine, and cryptorhynchine weevils (6, 15, 24); *Sodalis*-allied endosymbionts associated with dryophthorid weevils of the genus *Sitophilus* (14); and so-called "D-clade" endosymbionts of dryophthorid weevils of the genera *Trigonotarsus* and *Diocarandra* (24). Meanwhile, the B-type sequences from *C. sikkimensis* populations clustered with the endosymbiont of the tsetse fly, *Sodalis glossinidius* (7), endosymbionts of *Sitophilus* grain weevils (14), and the endosymbiont of the *Columbicola* pigeon louse (10).

Analysis of bacterial *groEL* gene sequences from *Curculio* weevils. A 0.9-kb segment of the bacterial *groEL* gene was amplified by PCR and cloned from DNA samples of *Curculio* weevils. The obtained 923-bp sequences were similar to each other (85.9% [793/923] to 94.0% [868/923] identities) between host species. BLAST searches with the *C. sikkimensis* sequence as the query retrieved the top hit sequence as the *groEL* gene sequence of *Blochmannia laevigatus*, an endosymbiont of the carpenter ant, *Camponotus laevigatus*, with relatively low sequence similarity (80.5% [743/923] identity; accession no. AY334434).

From DNA samples of *C. sikkimensis* wherein the A-type and B-type 16S rRNA gene sequences were identified, a 1.3-kb *groEL* gene segment was amplified by PCR with *Sodalis*-targeted primers, cloned, and sequenced. The obtained 1,289-bp sequence retrieved the BLAST top hit as an endosymbiont of the dryophthorid grain weevil *Sitophilus oryzae* with high sequence similarity (98.9% [1,274/1,289] identity; accession no. AF005236). The two types of bacterial *groEL* gene sequences from *C. sikkimensis* showed relatively low sequence similarity (77.2% [713/923] identity).

These sequences were subjected to molecular phylogenetic analysis with *groEL* gene sequences of gammaproteobacterial representatives (see Fig. S1 in the supplemental material). The former sequences formed a monophyletic group with high statistical supports. The bacterial clade was allied to neither *Nardonella* nor *Sodalis*. Meanwhile, the latter sequences clustered with *Sodalis glossinidius* and the *Sitophilus* endosymbiont.

Prevalence of the symbionts in *C. sikkimensis* populations. We examined the prevalence of the novel bacterial gene sequences and the *Sodalis*-allied gene sequences in three populations of *C. sikkimensis* by diagnostic PCR. The novel bacteria exhibited consistently high infection frequencies: 100% (30/30) in Tsukuba, 87% (26/30) in Hacchodaira, and 100% (30/30) in Shohbara. On the other hand, the *Sodalis*-allied bacteria were of low infection frequencies: 23% (7/30) in Tsukuba, 0% (0/30) in Hacchodaira, and 47% (14/30) in Shohbara. In the diagnostic PCR experiments described above, the results with the 16S rRNA gene were perfectly concordant with those with the

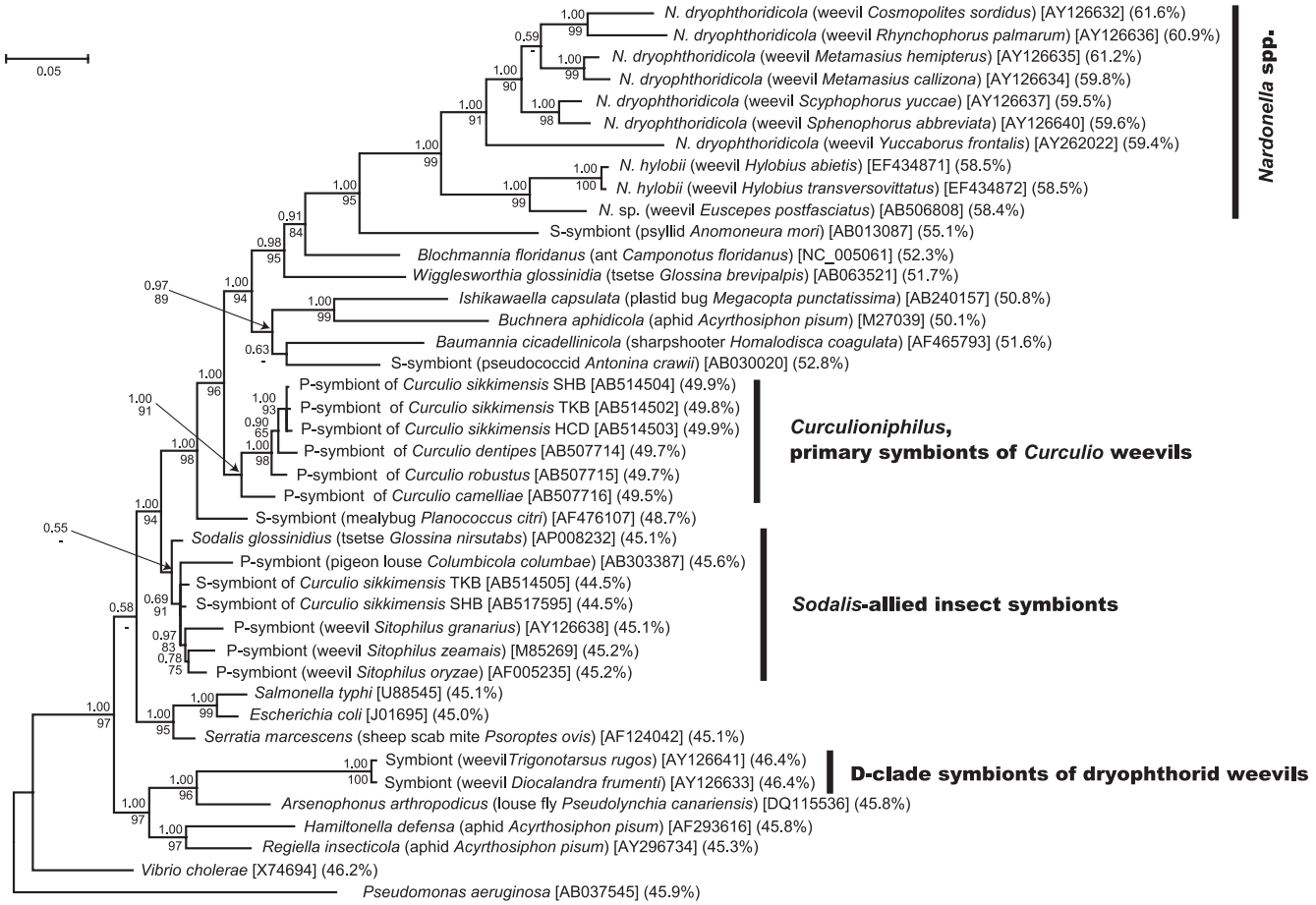

FIG. 1. Molecular phylogenetic analysis of the endosymbionts of *Curculio* weevils on the basis of 16S rRNA gene sequences. A Bayesian tree inferred from 1,180 unambiguously aligned nucleotide sites is shown, whereas a maximum likelihood analysis gave substantially the same topology (data not shown). Posterior probabilities for the Bayesian analysis and bootstrap values (>50%) for the maximum likelihood analysis are shown above and below the nodes, respectively. Sequence accession numbers and AT contents of the nucleotide sequences are in brackets and parentheses, respectively. As for insect endosymbionts, name of the host insect is also indicated in parentheses. P-symbiont, primary symbiont; S-symbiont, secondary symbiont.

groEL gene. Notably, the 16S rRNA gene sequence of *Nardonella* was detected from none of the insects (Table 1).

Localization and morphology of the novel symbiont in *C. sikkimensis*. Dissected tissues of larvae and adults of *C. sikkimensis* were subjected to whole-mount in situ hybridization targeting 16S rRNA of the novel symbiont. In larvae, the symbiont signals were localized in a bacteriome located at the posterior end of a voluminous stomach-like midgut region (Fig. 2B). The bacteriome was a loose assemblage of a number of bacteriocytes surrounding the outside of a tubular section of the midgut (Fig. 2C and D). Numerous filamentous bacteria were harbored in the cytoplasm of the bacteriocytes (Fig. 2E). Freshly smeared bacteriocyte specimens showed the dimensions of the bacterial cells as 14.1 ± 2.8 μm in length and 0.6 ± 0.1 μm in width (mean ± standard deviation [SD]; $n = 16$) (Fig. 2G). Electron microscopy revealed that the symbiont cells had a reduced cell wall and were present in the cytoplasm without being encased in a host-derived membrane (Fig. 3A and B). In adult females, the symbiont signals were detected in the germaria located at the tip of the ovarioles (Fig. 2F).

Accelerated molecular evolution in the novel symbiont from *Curculio* weevils. The evolutionary rates of the 16S rRNA and *groEL* gene sequences in the lineage of the novel symbionts of *Curculio* weevils were significantly higher than those in the lineages of free-living gammaproteobacteria, respectively. Meanwhile, the evolutionary rates in the *Sodalis*-allied symbionts of *Curculio* weevils were not significantly different from those of free-living gammaproteobacteria (Table 2).

AT-biased nucleotide composition in genes of the novel symbiont from *Curculio* weevils. The 16S rRNA gene sequences constituting the novel symbiont clade exhibited AT contents ranging from 49.5% to 49.9%, which were remarkably higher than the AT contents of free-living gammaproteobacteria of around 45%. On the other hand, the 16S rRNA gene sequences of the *Sodalis*-allied symbiont from *C. sikkimensis* were 44.5% AT, which was similar to the values in free-living gammaproteobacteria (Fig. 1).

The *groEL* gene sequences representing the novel symbiont clade exhibited AT contents ranging from 59.8% to 61.4%, which were drastically higher than the AT contents of free-

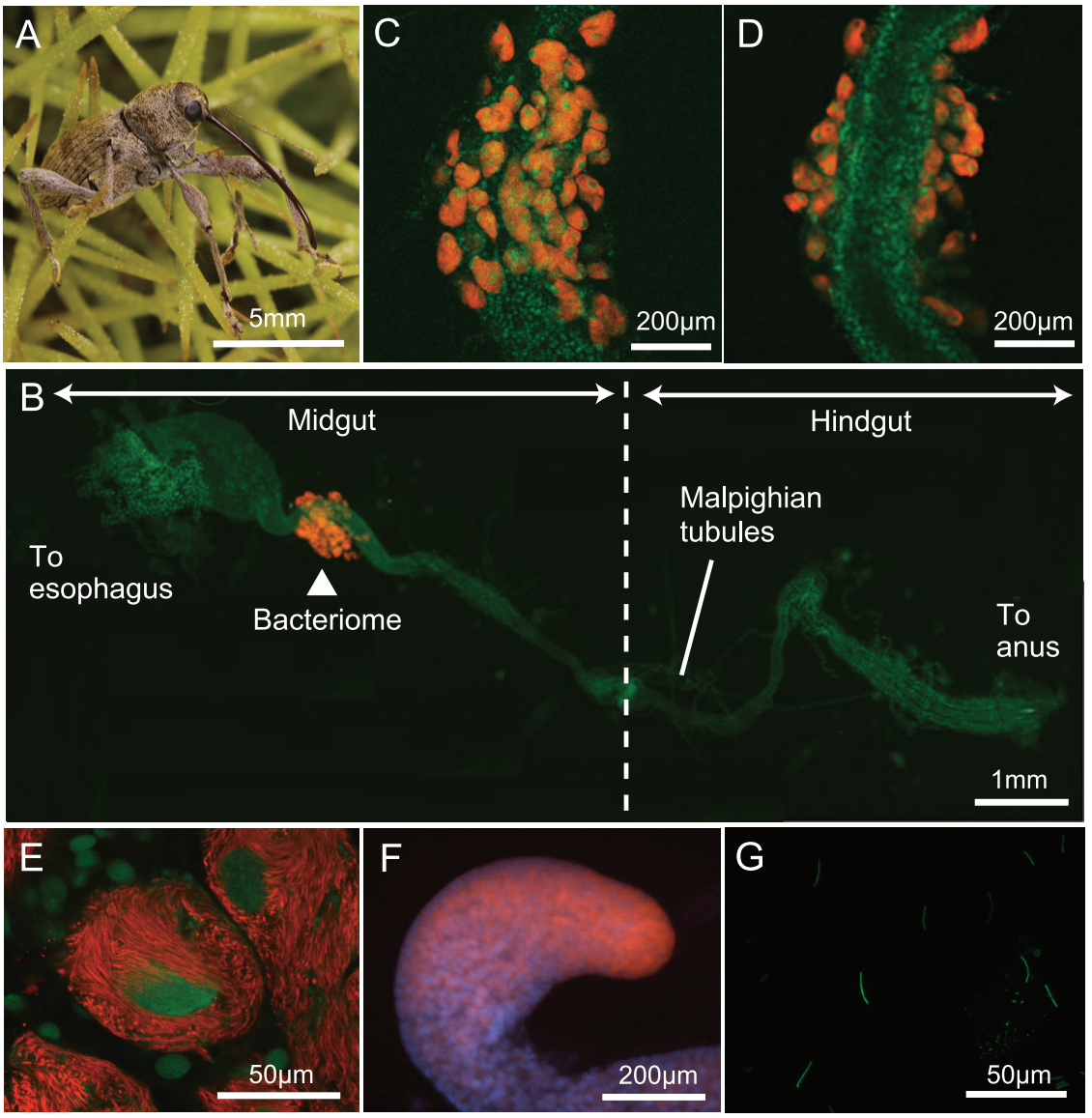

FIG. 2. (A) An adult insect of *C. sikkimensis*. (B to F) Localization of the primary symbiont in dissected tissues of *C. sikkimensis*. Red and green signals indicate 16S rRNA of the primary symbiont and nuclear DNA of the insect cells, respectively. (B) Location of the bacteriome visualized on a dissected intestine of a final instar larva; (C and D) enlarged images of the bacteriome, wherein many bacteriocytes loosely aggregate and surround the midgut tube; (E) an enlarged image of the bacteriocytes, whose cytoplasm is filled with filamentous bacterial cells; (F) the apex of an ovary dissected from an adult female, wherein the symbiont signals are seen; (G) freshly isolated symbiont cells stained with SYTOX green.

living gammaproteobacteria, ranging from 43% to 47%. Meanwhile, the *groEL* gene sequence of the *Sodalis*-allied symbiont from *C. sikkimensis* was 44.0% AT, which was comparable to the values in free-living gammaproteobacteria (see Fig. S1 in the supplemental material).

DISCUSSION

We found herein a novel group of bacterial endosymbionts from the weevil genus *Curculio*, which represents the primary symbiont of *Curculio* weevils. A well-defined gammaproteobacterial clade, *Nardonella* spp., comprises the ancient endosymbiont lineage associated with diverse weevils representing the Dryophthoridae, the Molytinae, and the Cryptorhynchinae, whose evolutionary origin is estimated to be some 125 million years ago (6, 15, 24). However, the *Nardonella*-weevil association has not been perfectly coherent. Symbiont replacements have occurred in several dryophthorid lineages, such as the clade of grain weevils, *Sitophilus* spp., and the clade of *Trigonatarsus-Diocarandra* (24). In a recent extensive molecular phylogenetic analysis of the Curculionoidea (27), molytine weevils and curculionine weevils were placed in the same clade, suggesting that the common ancestor of *Curculio* weevils was also associated with *Nardonella*. Hence, it seems likely that the symbiont replacement from *Nardonella* to the primary symbiont occurred in an ancestor of the *Curculio* weevils.

The ancestral weevil endosymbionts of the *Nardonella* spp.

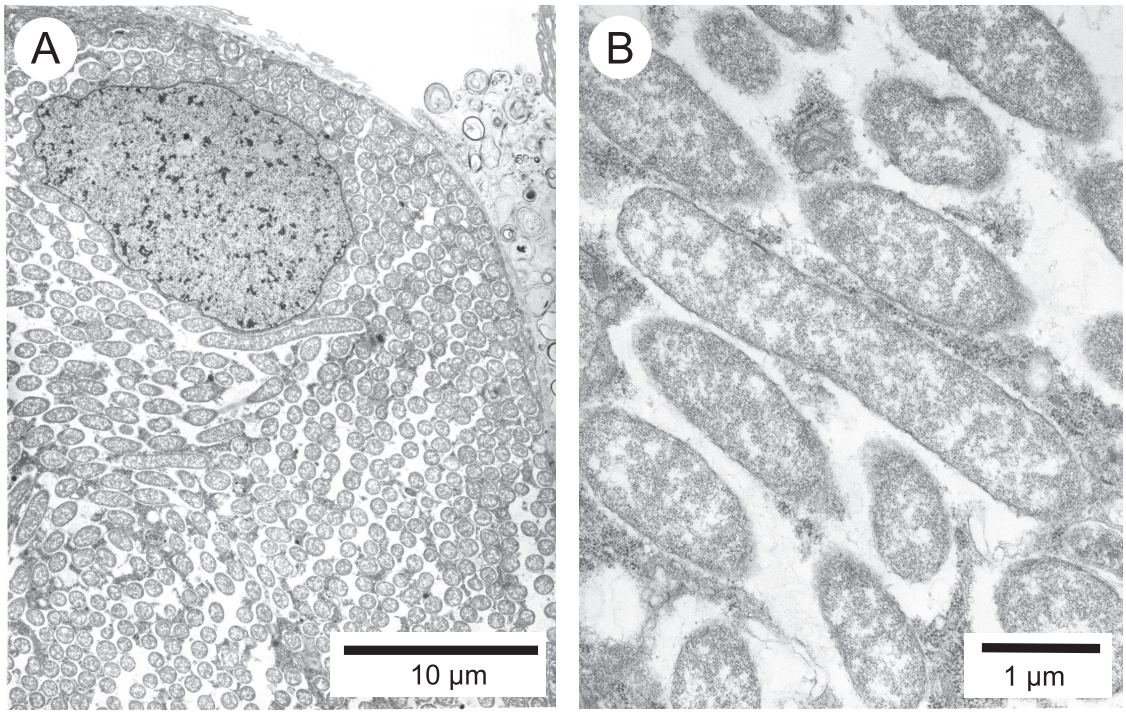

FIG. 3. Transmission electron microscopy of the bacteriocyte of *C. sikkimensis*. (A) A bacteriocyte, whose cytoplasm is full of slender bacterial cells; (B) an enlarged image of the symbiont cells.

are harbored in a U- or ring-shaped large bacteriome at larval stages. The bacteriome is a well-defined organ consisting of many bacteriocytes surrounding the alimentary tract at the foregut-midgut junction (5, 6, 31). In *Sitophilus*, *Trigonotarsus*, and *Diocalandra* species in which *Nardonella* has been replaced by different bacteria, the bacteriome remains in the same location and exhibits a similar histological configuration (5, 31). Hence, upon these symbiont replacement events, the symbiotic bacteria have been taken over while the symbiotic organ has been conserved. In the *Curculio* weevils, on the other hand, no bacteriome was present at the foregut-midgut junction, but, strikingly, a bacteriome of quite different histological configuration was found at the posterior end of a stomach-like midgut region (Fig. 2). Interestingly, the symbiont replacement event

TABLE 2. A relative-rate test for comparing the molecular evolutionary rates of 16S rRNA and *groEL* gene sequences between the lineages of the primary and secondary symbionts of *Curculio* weevils and their free-living relatives

Gene	Lineage 1	Lineage 2	Outgroup	K_1[a]	K_2[b]	Difference of distance between K_1 and K_2	K_1/K_2 ratio	P value[c]
16S rRNA gene of the primary symbionts ("*Ca.* Curculioniphilus")	P-symbionts of *Curculio* spp.[d]	*Escherichia coli* and *Salmonella enterica* serovar Typhi[e]	*V. cholerae*[f]	0.059	0.041	0.018	1.4	0.049
groEL gene of the primary symbionts ("*Ca.* Curculioniphilus")	P-symbionts of *Curculio* spp.[g]	*E. coli* and *S.* Typhi[h]	*V. cholerae*[i]	0.080	0.019	0.061	4.2	3.4×10^{-6}
16S rRNA gene of the secondary symbionts (*Sodalis*-allied)	S-symbionts of *Curculio* spp.[j]	*E. coli* and *S.* Typhi[e]	*V. cholerae*[f]	0.029	0.031	−0.002	0.94	0.82
groEL gene of the secondary symbionts (*Sodalis*-allied)	S-symbionts of *Curculio* spp.[k]	*E. coli* and *S.* Typhi[h]	*V. cholerae*[i]	0.030	0.014	0.016	2.1	0.066

[a] Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
[b] Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
[c] *P* values were generated using the program RRTree (35).
[d] P-symbiont of *C. sikkimensis* (AB514502), *C. camelliae* (AB507716), *C. dentipes* (AB507714), and *C. robustus* (AB507715).
[e] *E. coli* (J01695) and *S.* Typhi (U88545).
[f] *V. cholerae* (X74694).
[g] P-symbiont of *C. sikkimensis* (AB514497) and *C. camelliae* (AB514498).
[h] *E. coli* (X07850) and *S.* Typhi (U01039).
[i] *V. cholerae* (CP000627).
[j] S-symbiont of *C. sikkimensis* (AB514505 and AB517595).
[k] S-symbiont of *C. sikkimensis* (AB514501).

in an ancestor of *Curculio* weevils entailed development of a different symbiotic organ, whose evolutionary origin comprises an enigma.

As for the primary symbiont of *Curculio* weevils, its high prevalence in host populations (Table 1), high AT contents (Fig. 1 and see Fig. S1 in the supplemental material) and accelerated molecular evolution (Table 2) suggest an intimate host-symbiont association over evolutionary time and also an important biological role of the symbiont for the host. *Curculio* weevils feed on acorns, seeds, or galls of their specific host plants at their larval stages (17). The symbiont may provide some nutritional components that are deficient in those plant parts, as many insect symbionts do for their hosts (8). Alternatively, the symbiont might be involved in detoxification of defense substances accumulated in those plant parts. Fagaceous acorns are known to contain high concentrations of tannins (40, 47). Some insect-induced plant galls also contain high levels of tannins, which were traditionally utilized for industrial production of gallotannin (2). Many plant seeds accumulate various toxic substances as antiherbivore agents (18, 49). In some wild mice that mainly feed on fagaceous acorns, their resistance to tannins was shown to be attributed to tannase-producing gut bacterial symbionts (41). Whether the primary symbiont of *Curculio* weevils plays a similar role deserves future studies.

When adult weevils collected from three natural populations of *C. sikkimensis* were examined by diagnostic PCR for the primary symbiont infection, the Tsukuba population and Shohbara population exhibited 100% infection frequencies, whereas the Hacchodaira population contained some uninfected insects, attaining 87% infection frequency (Table 1). This result suggests that the primary symbiont may not always be essential for *C. sikkimensis*. In the grain weevils, *Sitophilus* spp., aposymbiotic insects are able to grow and reproduce while suffering several defects, including retarded growth and lower fecundity than symbiotic insects (14). In the alydid bug *Riptortus pedestris* (= *R. clavatus*), symbiont-free insects become adults and lay viable eggs, but their body size is smaller than that of symbiont-infected insects (22). Similarly, the primary symbiont of *Curculio* weevils might be such a "beneficial but not essential" endosymbiont. Considering that the diagnostic PCR analysis was performed on adult insects only, the primary symbiont might be essential for larvae but not for adults of *Curculio* weevils. Alternatively, the primary symbiont might be conditionally needed for *Curculio* weevils, depending on ecological, physiological, and/or environmental factors the insects have experienced. To address which of these hypotheses is the most appropriate, experimental studies on symbiotic and aposymbiotic *Curculio* weevils are needed.

On account of the distinct genetic, phylogenetic, and microbiological traits described above, we propose the designation "*Candidatus* Curculioniphilus buchneri" for the primary symbiotic bacteria identified from the weevils of the genus *Curculio*. The generic name refers to the affinity to *Curculio* weevils. The specific name honors Paul Buchner, who first described the endosymbiotic bacteria of *Curculio* weevils (4).

In addition to the primary symbiont "*Ca.* Curculioniphilus," we identified *Sodalis*-allied gammaproteobacterial endosymbionts from *C. sikkimensis* (Fig. 1 and see Fig. S1 in the supplemental material). The diagnostic PCR survey revealed relatively low infection frequencies of the *Sodalis*-allied bacteria: 23% in the Tsukuba population, 0% in the Hacchodaira population, and 47% in the Shohbara population (Table 1). Meanwhile, the bacterial genes were not detected from the other *Curculio* species, although the samples examined in this study are limited in number (Table 1). The bacterial gene sequences exhibited neither AT-biased nucleotide composition (Fig. 1 and Fig. S1) nor accelerated molecular evolution (Table 2). On the basis of these results, we conclude that the *Sodalis*-allied bacteria represent the secondary symbiont of *C. sikkimensis*.

As mentioned above, in the grain weevils of the genus *Sitophilus*, the ancient endosymbiont *Nardonella* has been lost and replaced by a *Sodalis*-allied endosymbiont (24), which exhibits bacteriome-specific localization and benefits the host's growth and reproduction (14). It appears plausible that the bacteriome-associated *Sodalis*-allied primary symbiont of *Sitophilus* spp. might have evolved from a *Sodalis*-allied secondary symbiont of weevils. In this context, it is of great interest how commonly *Sodalis*-allied facultative symbionts are found in the diversity of extant weevils. Comparative studies on the *Sodalis*-allied primary symbiont of *Sitophilus* spp. and the *Sodalis*-allied secondary symbiont of *C. sikkimensis* would provide insights into how a less-specialized endosymbiont has established the bacteriome-specific localization and the mutualistic association.

ACKNOWLEDGMENTS

We thank M. Higaki and the laboratory of pear/chestnut horticulture, National Institute of Fruit Tree Science, for technical advice on rearing of *C. sikkimensis*; Y. Higashiura and Y. Sato for information on sampling localities; K. Fuse, I. Hanya, M. Hanya, and S. Yokota for weevil samples; S. Hanada for bacterial nomenclature; and H. Anbutsu, M. Kutsukake, Y. Matsuura, K. Tanaka, and T. Tsuchida for advice on laboratory experiments.

This study was funded by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and also by a grant-in-aid (no. 204352) from the Ministry of Education, Science, Sports and Culture, Japan. H.T. was supported by the Japan Society for the Promotion of Science (JSPS) postdoctoral fellowship for young scientists.

REFERENCES

1. **Akman, L., A. Yamashita, H. Watanabe, K. Oshima, T. Shiba, M. Hattori, and S. Aksoy.** 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. Nat. Genet. **32:**402–407.
2. **Bernays, E. A., G. C. Driver, and M. Bilgener.** 1989. Herbivores and plant tannins. Adv. Ecol. Res. **19:**263–302.
3. **Bourtzis, K., H. Braig, and T. Karr.** 2003. Cytoplasmic incompatibility, p. 217–246. *In* K. Bourtzis and T. Miller (ed.), Insect symbiosis. CRC, Boca Raton, FL.
4. **Buchner, P.** 1933. Studien an intracellularen symbionten. Zoomorphology **26:**709–777.
5. **Buchner, P.** 1965. Endosymbionts of animals with plant microorganisms. Interscience, New York, NY.
6. **Conord, C., L. Despres, A. Vallier, S. Balmand, C. Miquel, S. Zundel, G. Lemperiere, and A. Heddi.** 2008. Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: additional evidence of symbiont replacement in the Dryophthoridae family. Mol. Biol. Evol. **25:**859.
7. **Dale, C., and I. Maudlin.** 1999. *Sodalis* gen. nov., and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans* morsitans. Int. J. Syst. Bacteriol. **49:**267–275.
8. **Douglas, A. E.** 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu. Rev. Entomol. **43:**17–37.
9. **Fukatsu, T.** 1999. Acetone preservation: a practical technique for molecular analysis. Mol. Ecol. **8:**1935–1945.
10. **Fukatsu, T., R. Koga, W. A. Smith, K. Tanaka, N. Nikoh, K. Sasaki-Fukatsu, K. Yoshizawa, C. Dale, and D. H. Clayton.** 2007. Bacterial endosymbiont of the slender pigeon louse, *Columbicola columbae*, allied to endosymbionts of grain weevils and tsetse flies. Appl. Environ. Microbiol. **73:**6660–6668.

11. **Fukatsu, T., and N. Nikoh.** 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl. Environ. Microbiol. **64:**3599–3606.
12. **Guindon, S., F. Lethiec, P. Duroux, and O. Gascuel.** 2005. PHYML Online: a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res. **33:**W557.
13. **Heddi, A., F. Lefebvre, and P. Nardon.** 1993. Effect of endocytobiotic bacteria on mitochondrial enzymatic activities in the weevil *Sitophilus oryzae* (Coleoptera, Curculionidae). Insect Biochem. Mol. Biol. **23:**403–411.
14. **Heddi, A., and P. Nardon.** 2005. *Sitophilus oryzae* L.: a model for intracellular symbiosis in the Dryophthoridae weevils (Coleoptera). Symbiosis **39:**1–11.
15. **Hosokawa, T., and T. Fukatsu.** *Nardonella* endosymbiont in the West Indian sweet potato weevil *Euscepes postfasciatus* (Coleoptera: Curculionidae). Appl. Entomol. Zool., in press.
16. **Hosokawa, T., Y. Kikuchi, M. Shimada, and T. Fukatsu.** 2007. Obligate symbiont involved in pest status of host insect. Proc. R. Soc. B **274:**1979–1984.
17. **Hughes, J., and A. P. Vogler.** 2004. Ecomorphological adaptation of acorn weevils to their oviposition site. Evolution **58:**1971–1983.
18. **Hulme, P., and C. W. Benkman.** 2002. Granivory, p. 132–154. *In* C. M. Herrera and O. Pellmyr (ed.), Plant-animal interactions: an evolutionary approach. Blackwell Publishing, Oxford, United Kingdom.
19. **Ihara, F., M. Toyama, M. Higaki, K. Mishiro, and K. Yaginuma.** 2009. Comparison of pathogenicities of *Beauveria bassiana* and *Metarhizium anisopliae* to chestnut pests. Appl. Entomol. Zool. **44:**127–132.
20. **Janson, E. M., J. O. Stireman III, M. S. Singer, and P. Abbot.** 2008. Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution **62:**997–1012.
21. **Kellner, R. L. L.** 2002. Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus* (Coleoptera: Staphylinidae). Insect Biochem. Mol. Biol. **32:**389–395.
22. **Kikuchi, Y., T. Hosokawa, and T. Fukatsu.** 2007. Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl. Environ. Microbiol. **73:**4308–4316.
23. **Koga, R., T. Tsuchida, and T. Fukatsu.** 2009. Quenching autofluorescence of insect tissues for in situ detection of endosymbionts. Appl. Entomol. Zool. **44:**281–291.
24. **Lefevre, C., H. Charles, A. Vallier, B. Delobel, B. Farrell, and A. Heddi.** 2004. Endosymbiont phylogenesis in the Dryophthoridae weevils: evidence for bacterial replacement. Mol. Biol. Evol. **21:**965–973.
25. **Maeto, K., and K. Ozaki.** 2003. Prolonged diapause of specialist seed-feeders makes predator satiation unstable in masting of *Quercus crispula*. Oecologia **137:**392–398.
26. **Mansour, K.** 1930. Preliminary studies on the bacterial cell mass (accessory cell mass) of *Calandra oryzae*: the rice weevil. Q. J. Microsc. Sci. **73:**421–436.
27. **McKenna, D. D., A. S. Sequeira, A. E. Marvaldi, and B. D. Farrell.** 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. Proc. Natl. Acad. Sci. U. S. A. **106:**7083–7088.
28. **Menu, F.** 1993. Strategies of emergence in the chestnut weevil *Curculio elephas* (Coleoptera, Curculionidae). Oecologia **96:**383–390.
29. **Montllor, C. B., A. Maxmen, and A. H. Purcell.** 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol. Entomol. **27:**189–195.
30. **Moran, N. A.** 2007. Symbiosis as an adaptive process and source of phenotypic complexity. Proc. Natl. Acad. Sci. U. S. A. **104:**8627.
31. **Nardon, P., C. Lefevre, B. Delobel, H. Charles, and A. Heddi.** 2002. Occurrence of endosymbiosis in Dryophthoridae weevils: cytological insights into bacterial symbiotic structures. Symbiosis **33:**227–241.
32. **Nardon, P., C. Louis, G. Nicolas, and A. Kermarrec.** 1985. Mise en évidence et éude des bactéries symbiotiques chez deux charançons parasites du bananier: *Cosmopolites sordidus* (GERMAR) et *Metamasius hemipterus* (L.) (Col. Curculionidae). Ann. Soc. Entomol. Fr. **21:**245–258.
33. **Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter.** 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. USA **100:**1803–1807.
34. **Pierantoni, U.** 1927. L'organo simbiotico nello sviluppo di *Calandra oryzae*. Rend. Reale Acad. Sci. Fis. Mat. Napoli **35:**244–250.
35. **Robinson-Rechavi, M., and D. Huchon.** 2000. RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. Bioinformatics **16:**296–297.
36. **Ronquist, F., and J. P. Huelsenbeck.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:**1572–1574.
37. **Scarborough, C. L., J. Ferrari, and H. C. J. Godfray.** 2005. Aphid protected from pathogen by endosymbiont. Science **310:**1781.
38. **Scheinert, W.** 1933. Symbiose und Embryonalentwicklung bei Rüsselkäfern. Z. Morphol. Oekol. Tiere **27:**76–128.
39. **Shigenobu, S., H. Watanabe, M. Hattori, Y. Sakaki, and H. Ishikawa.** 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature **407:**81–86.
40. **Shimada, T.** 2001. Nutrient compositions of acorns and horse chestnuts in relation to seed-hoarding. Ecol. Res. **16:**803–808.
41. **Shimada, T., T. Saitoh, E. Sasaki, Y. Nishitani, and R. Osawa.** 2006. Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. J. Chem. Ecol. **32:**1165–1180.
42. **Tanabe, A. S.** 2007. kakusan: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. Mol. Ecol. Notes **7:**962–964.
43. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
44. **Toju, H., and T. Sota.** 2006. Adaptive divergence of scaling relationships mediates the arms race between a weevil and its host plant. Biol. Lett. **2:**539–542.
45. **Tsuchida, T., R. Koga, and T. Fukatsu.** 2004. Host plant specialization governed by facultative symbiont. Science **303:**1989.
46. **Werren, J. H., L. Baldo, and M. E. Clark.** 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. **6:**741–751.
47. **Xiao, Z., M. K. Harris, and Z. Zhang.** 2007. Acorn defenses to herbivory from insects: implications for the joint evolution of resistance, tolerance and escape. For. Ecol. Manag. **238:**302–308.
48. **Yoshida, N., K. Oeda, E. Watanabe, T. Mikami, Y. Fukita, K. Nishimura, K. Komai, and K. Matsuda.** 2001. Protein function: chaperonin turned insect toxin. Nature **411:**44.
49. **Zangerl, A. R., and M. R. Berenbaum.** 2005. Increase in toxicity of an invasive weed after reassociation with its coevolved herbivore. Proc. Natl. Acad. Sci. U. S. A. **102:**15529–15532.